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Increased Glutathione Biosynthesis Plays a Role in Nickel Tolerance in Thlaspi Nickel Hyperaccumulators [W]

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Worldwide more than 400 plant species are now known that hyperaccumulate various trace metals (Cd, Co, Cu, Mn, Ni, and Zn), metalloids (As) and nonmetals (Se) in their shoots. Of these, almost one-quarter are Brassicaceae family members, including numerous Thlaspi species that hyperaccumulate Ni up to 3% of there shoot dry weight. We observed that concentrations of glutathione, Cys, and *O*-acetyl-L-serine (OAS), in shoot tissue, are strongly correlated with the ability to hyperaccumulate Ni in various Thlaspi hyperaccumulators collected from serpentine soils, including *Thlaspi goesingense*, *T. oxyceras*, and *T. rosulare*, and nonaccumulator relatives, including *T. perfoliatum*, *T. arvense*, and *Arabidopsis thaliana*. Further analysis of the Austrian Ni hyperaccumulator *T. goesingense* revealed that the high concentrations of OAS, Cys, and GSH observed in this hyperaccumulator coincide with constitutively high activity of both serine acetyltransferase (SAT) and glutathione reductase. SAT catalyzes the acetylation of L-Ser to produce OAS, which acts as both a key positive regulator of sulfur assimilation and forms the carbon skeleton for Cys biosynthesis. These changes in Cys and GSH metabolism also coincide with the ability of *T. goesingense* to both hyperaccumulate Ni and resist its damaging oxidative effects. Overproduction of *T. goesingense* SAT in the nonaccumulator Brassicaceae family member Arabidopsis was found to cause accumulation of OAS, Cys, and glutathione, mimicking the biochemical changes observed in the Ni hyperaccumulators. In these transgenic Arabidopsis, glutathione concentrations strongly correlate with increased resistance to both the growth inhibitory and oxidative stress induced effects of Ni. Taken together, such evidence supports our conclusion that elevated GSH concentrations, driven by constitutively elevated SAT activity, are involved in conferring tolerance to Ni-induced oxidative stress in Thlaspi Ni hyperaccumulators.

INTRODUCTION

The observation that particular plants contain high concentrations of certain metals dates back to the origins of biogeochemical prospecting. As early as 1865, F. Risse, a German botanist, observed that leaves of certain plant species growing in soils naturally enriched in Zn contained extraordinarily high levels of this element, up to 1.5% of the shoot dry weight (Sachs, 1865). Fifty years later, studies in the U.S. implicated Se as the plant component responsible for alkali disease in range animals. This observation led to the discovery of plants, notably of the genus Astragalus, capable of accumulating up to 0.6% Se in dry shoot biomass (Byers, 1935). Shortly thereafter, two Italian botanists discovered plants that accumulate Ni (Minguzzi and Vergnano, 1948). They observed that dried leaves of *Alyssum bertolonii* growing on Ni enriched ultramafic (serpentine) soils contained ~1% Ni, more than 100 to 1000 times more than other plants growing nearby. In a landmark article on the study of metal accumulation in plants, Brooks and coworkers determined the Ni concentrations in more than 2000 herbarium specimens (Brooks et al., 1977). Based on this information, Brooks first refined the term hyperaccumulator, previously introduced by Jaffré and coworkers to describe plants that contain >1000 $\mu g\ g^{-1}$ (0.1%) Ni in their dried leaves, a concentration at least an order of magnitude higher than Ni levels in nonaccumulator species (Jaffré et al., 1976; Brooks et al., 1977). Presently, at least 45 plant families are known to contain numerous metal hyperaccumulating species (Reeves and Baker, 2000). A better understanding of the molecular and biochemical basis of this metal accumulation process should lead to development of both mineral nutrient fortified crops and plants suitable for phytoremediation of metal-polluted soils and waters (Guerinot and Salt, 2001).

To develop a practical genetic model system for dissecting the mechanistic basis of metal hyperaccumulation, we have been studying numerous biannual Ni and Zn hyperaccumulators from the Brassicaceae, including members of the Cochlearia and Thlaspi genera, collected from both serpentine and mine sites in Austria, France, Greece, Turkey, and the U.S. (Peer et al., 2003). To complement these broad-based studies, we have also focused on *Thlaspi goesingense* Hálácsy (Brassicaceae),

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a hyperaccumulator species found growing on naturally Ni enriched serpentine soils in Redschlag, Austria, where it accumulates up to 1.2% of its shoot dry weight as Ni (Reeves and Brooks, 1983; Krämer et al., 1997; Wenzel and Jockwer, 1999). This plant makes an attractive biochemical system for several reasons, including its ability to hyperaccumulate Ni under laboratory conditions (Krämer et al., 1997), its 86% identity at the genetic level (average of ITS1 and ITS2) to the model plant *Arabidopsis thaliana*, its compact, robust growth habit, and its ability to be genetically transformed by floral dipping using *Agrobacterium tumefaciens* (Peer et al., 2003). Our previous studies with *T. goesingense* have revealed that Ni hypertolerance is essential for Ni hyperaccumulation (Krämer et al., 1997), and enhanced vacuolar storage of Ni, as a Ni^{2+}-organic acid complex, is a major determinant of this hypertolerance (Krämer et al., 2000; Küpper et al., 2001; Persans et al., 2001). However, vacuolar compartmentalization is not the only mechanism involved in Ni hypertolerance in the hyperaccumulator because significant amounts of cellular Ni also accumulate outside the vacuole (Krämer et al., 2000).

The recent identification of Ni^{2+} complexed to the high affinity metal chelate nicotianamine in the Ni/Zn hyperaccumulator *T. caerulescens* (Vacchina et al., 2003) suggests that nicotianamine plays an important role in detoxification of extravacuolar Ni in hyperaccumulating plants. Constitutive overproduction of nicotianamine and the enzyme responsible for its biosynthesis, nicotianamine synthase, in *T. caerulescens* and the related hyperaccumulator *A. halleri* (Vacchina et al., 2003; Becher et al., 2004; Weber et al., 2004) strongly supports such a conclusion and suggests that nicotianamine overproduction is one general mechanism underlying Ni/Zn hyperaccumulation in the Brassicaceae family.

Here, we present evidence that *T. goesingense* also tolerates this elevated, nonvacuolar pool of Ni, through enhanced oxidative stress resistance, mediated by enhanced Cys and GSH biosynthesis and accumulation, achieved by constitutively elevated serine acetyltransferase (SAT) activity producing an elevated concentration of its product, *O*-acetyl-L-serine (OAS), and glutathione reductase maintaining high levels of GSH. Constitutive activation of SAT provides an ideal mechanism for increased biosynthesis of Cys and glutathione because SAT has been termed the bottleneck for Cys and glutathione synthesis in plants (Hesse et al., 2000), with its product, OAS, both upregulating sulfate uptake and reduction (Leustek, 2002; Hirai et al., 2003) and providing the carbon skeleton for sulfide assimilation into Cys (Hell, 1997). Identification of a positive correlation between OAS, Cys, and glutathione concentrations and Ni hyperaccumulation in various species of Thlaspi hyperaccumulator and nonaccumulator relatives suggests that this mechanism of Ni tolerance identified in *T. goesingense* represents a more general mechanism of Ni tolerance in Thlaspi Ni hyperaccumulators.

RESULTS

Correlation among Shoot OAS, Cys, Glutathione, and Ni Hyperaccumulation

Various hyperaccumulator and nonaccumulator species of the Thlaspi and Arabidopsis genera were grown and shoot material sampled and analyzed for OAS, Cys, and total glutathione. In parallel, plants were grown in Ni-enriched soil and analyzed for shoot Ni (Peer et al., 2003). Plotting shoot OAS against shoot Ni concentrations (Figure 1A) revealed a strong positive relationship between OAS and the ability to accumulate Ni in plants known to hyperaccumulate this metal in their native environment. Nonaccumulators, including *T. perfoliatum*, *T. arvense*, *T. montanum* var fendleri, *A. lyrata*, and *A. thaliana*, showed uniformly low OAS concentrations. Remarkably, the concentration of OAS in shoot tissue also showed a strong positive relationship with the concentration of Ni accumulated by these plants in their natural habitat (Figure 1B). Further analysis of hyperaccumulator and nonaccumulator species also revealed a strong positive relationship between both shoot Cys and glutathione concentrations and Ni hyperaccumulation ability (Figures 1C to 1F).

To further study the role of OAS, Cys, and glutathione in Ni hyperaccumulation, the Ni hyperaccumulator *T. goesingense* and the nonaccumulating relative *A. thaliana* were chosen as a comparative pair. Comparison of the OAS concentrations in shoot material from *T. goesingense* and *A. thaliana*, grown under identical conditions and harvested at the same developmental stage, revealed a 150-fold higher concentration in the hyperaccumulator (Figure 2B) when the plants where grown in the absence of Ni. Such observations are consistent with the differences in OAS observed between numerous hyperaccumulators and various nonaccumulating relatives including *A. thaliana*, *A. lyrata*, *T. arvense*, *T. perfoliatum*, and *T. montanum* var fendleri when grown in soil (Figure 1). Furthermore, SAT activity was found to be 5.5-fold higher in *T. goesingense* compared with the nonaccumulator *A. thaliana* (Figure 2 A), consistent with the elevated OAS. Exposure to 100 μM Ni in the growth media for 8 weeks, simulating the chronic Ni exposure normally encountered by the hyperaccumulator in the field, produced no significant change (Student's *t* test, $P > 0.05$) in OAS or SAT activity in *T. goesingense*. However, exposure of the nonaccumulator *A. thaliana* to 10 μM Ni for 10 d, the highest tolerable Ni exposure conditions, produced a significant (Student's *t* test, $P < 0.05$) reduction in SAT activity from 19 to 6 pmol OAS mg^{-1} total protein min^{-1}. OAS provides the carbon skeleton for the biosynthesis of Cys. Quantification of Cys in the hyperaccumulator and nonaccumulator revealed a 4.5-fold higher Cys concentration in *T. goesingense* (Figure 2C). This higher Cys concentration was also reflected in a 1.5- to 1.6-fold elevation of total GSH (Figure 2D) and a doubling of the GSH/GSSG ratio (Figure 2E). Elevated levels of reduced GSH in the hyperaccumulator (Figure 2E) are also accompanied by a twofold increase in glutathione reductase activity (Figure 2F) and a significant (Student's *t* test, $P < 0.01$) 50% increase in catalase activity.

Resistance to Ni in *T. goesingense* and *A. thaliana*

After 18 d of growth on medium containing 100 μM Ni, *T. goesingense* showed no visible symptoms of Ni toxicity (Figures 3A and 3B), whereas *A. thaliana* suffered significant loss of chlorophyll as well as a major reduction in shoot and root growth (Figures 3A and 3B). Oxidative damage after growth in the presence of Ni was also measured in *T. goesingense* and *A. thaliana*, as both total lipid peroxidation (thiobarbituric acid

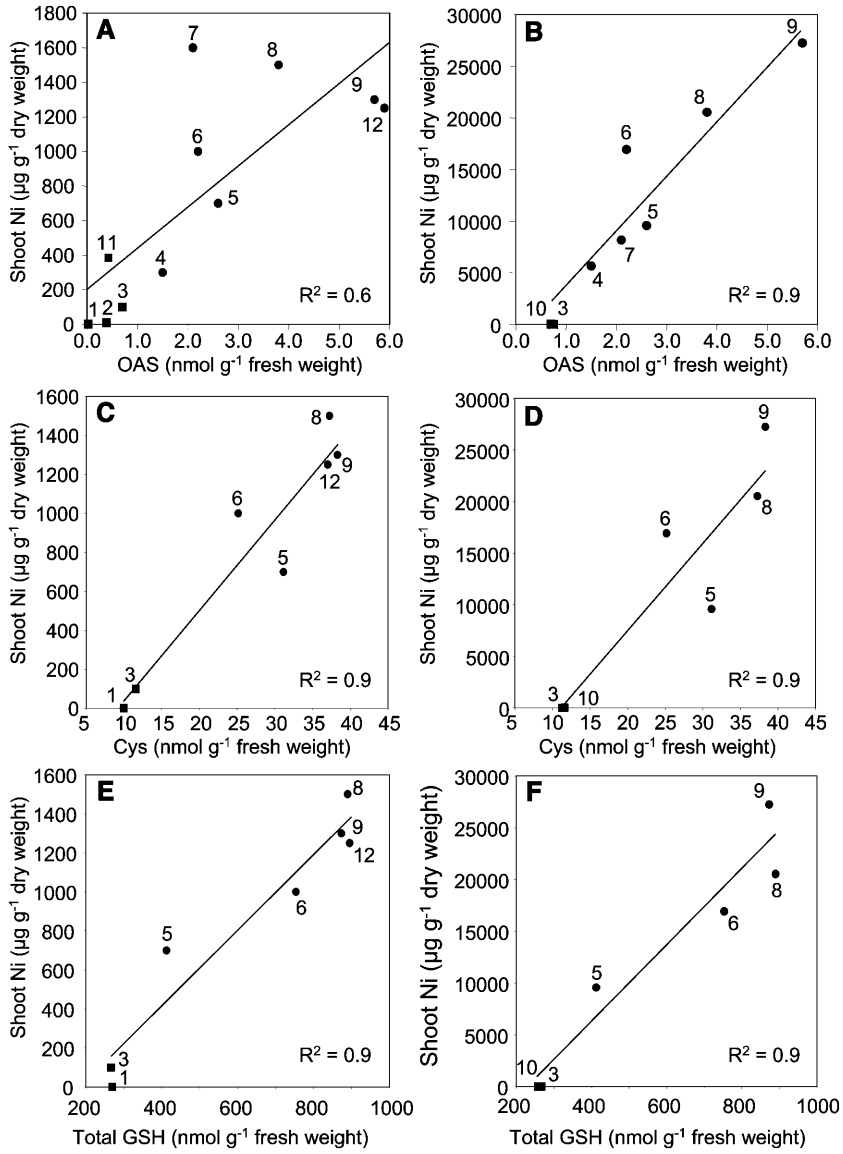

Figure 1. Shoot OAS, Cys, Glutathione, and Ni Concentrations in Various Hyperaccumulator and Nonaccumulator Brassicaceae Species.

Ni hyperaccumulator (circles) and nonaccumulator (squares) species with shoot OAS, Cys, and glutathione measured in greenhouse grown plants and plotted against shoot Ni from plants grown in the greenhouse (**[A]**, **[C]**, and **[E]**) or shoot Ni measured in plants from their native habitat (**[B]**, **[D]**, and **[F]**). Shoot Ni concentrations in greenhouse grown plants 1 to 3 and 5 to 12 as reported in Peer et al. (2003). Shoot Ni in plants 5, 7, and 10 from their native habitat as reported in Wenzel and Jockwer (1999) and Reeves et al. (2001); all other data from this study. Numbers represent different species and accessions as follows. *A. thaliana* (1), *A. lyrata* (2), *T. perfoliatum* (3), *T. montanum* var montanum (4), *T. goesingense* (Redschlag) (5), *T. oxyceras* (Zorkun yayla) (6), *T. caerulescens* (Puy de Wolf) (7), *T. oxyceras* (Osmaniye) (8), *T. rosulare* (Içel A) (9), *T. arvense* (Col de Gleize) (10), *T. montanum* var fendleri (Schultz pass) (11), and *T. oxyceras* (Refahiye Imranlý) (12). Trend line and R^2 represent regression analysis.

reactive species [TBARS]) in shoot tissue (Figure 3C) and presence of reactive oxygen species (ROS) in root tips (Figures 3D to 3F). Growth on media containing Ni for 8 d produced no significant increase in total lipid peroxidation in shoot tissue of the hyperaccumulator (Figure 3C). In comparison, total lipid peroxidation in shoot tissue of the nonaccumulator *A. thaliana* increased 800% (Figure 3C). Consistent with this, after exposure to Ni, the majority of root tips in the hyperaccumulator showed no reactive oxygen–induced fluorescence (Figures 3D and 3E). By contrast, >90% of the root tips of *A. thaliana* showed high levels of reactive oxygen–induced fluorescence (Figures 3D and 3F).

Speciation of Ni in *T. goesingense* and *A. thaliana*

X-ray absorption spectroscopy (XAS) was used to obtain information on the in planta Ni speciation in the Ni

hyperaccumulator *T. goesingense*. Previously, we have observed that Ni speciation in hydroponically grown *T. goesingense* shoots is dominated by Ni-O coordination with no significant Ni-S interactions (Krämer et al., 2000). To address the possibility that *T. goesingense* may behave differently during shorter term Ni exposure in hydroponics, compared with natural field exposure, we collected and rapidly froze *T. goesingense* shoot tissue in the field at Redschlag, Austria. Analysis of the Ni speciation in this tissue, using XAS, confirmed Ni-O to be the predominant coordination form of Ni with no significant presence of Ni-S (Figure 4, Table 1). A lack of significant Ni-S coordination was also supported by the absence of phytochelatin accumulation after Ni exposure in the hyperaccumulator (data not shown).

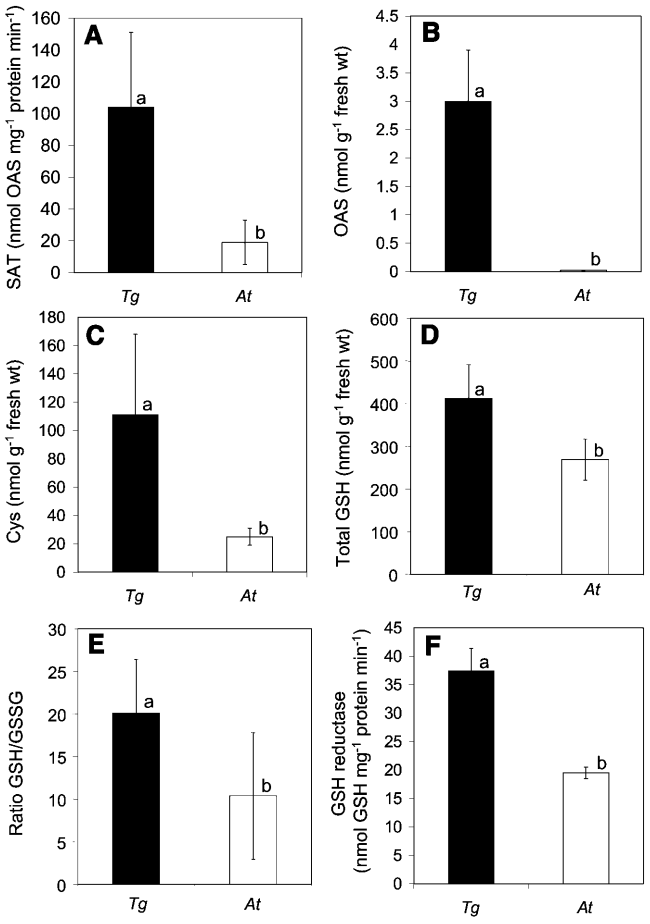

Figure 2. Comparison of Biochemical Changes in the Hyperaccumulator *T. goesingense* and the Nonaccumulator *A. thaliana* Grown in an Artificial Soil Mix in the Absence of Ni^{2+}.

Quantification of SAT activity **(A)**, OAS **(B)**, Cys **(C)**, total glutathione **(D)**, ratio reduced to oxidized glutathione **(E)**, and GSH reductase activity **(F)**. Data represent averages ($n = 5 – 16$) ± SD. Different lower case letters (a and b) represent significant differences (Student's *t* test, $P < 0.03$). Black bars, *T. goesingense*; white bars, *A. thaliana*.

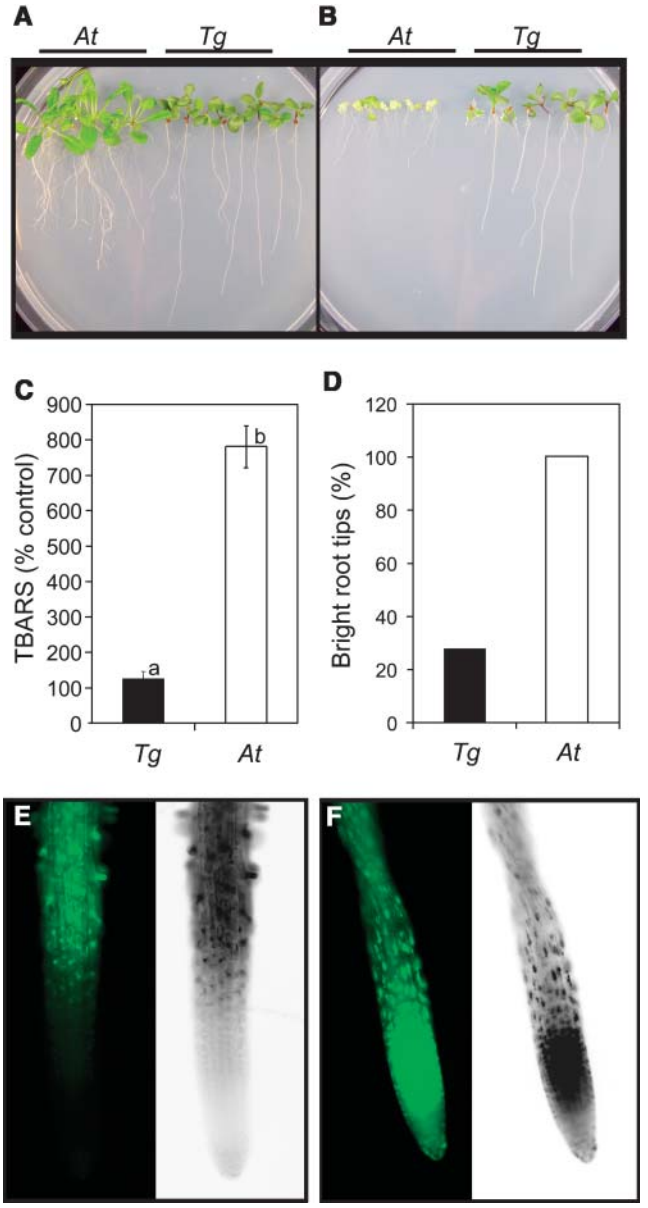

Figure 3. Comparison of the Ni Resistance of Hyperaccumulator *T. goesingense* and Nonaccumulator *A. thaliana*.

T. goesingense (*Tg*) and *A. thaliana* (*At*) plants were grown together on half-strength MS agar plates **(A)** or plates containing 100 μM Ni^{2+} **(B)** for 18 d from imbibition. Shoot lipid peroxidation **(C)** measured as TBARS in the hyperaccumulator *T. goesingense* and the nonaccumulator *A. thaliana* after exposure to 125 μM Ni for 4 d. Data represent averages ($n = 4$) ± SD. Different lower case letters (a and b) represent significant differences (Student's *t* test, $P < 0.01$). ROS **(D)** measured as fluorescence of 5-(and-6)-carboxy-2',7'-dichloro-dihyrofluorescein diacetate (carboxy-H2DCFDA) in root tips of *T. goesingense* and *A. thaliana* after exposure to 125 μM Ni for 8 d, presented as a percentage of brightly fluorescent root tips (*T. goesingense*, $n = 18$; *A. thaliana*, $n = 83$) or fluorescent images (positive and negative) of root tips of *T. goesingense* **(E)** and *A. thaliana* **(F)**.

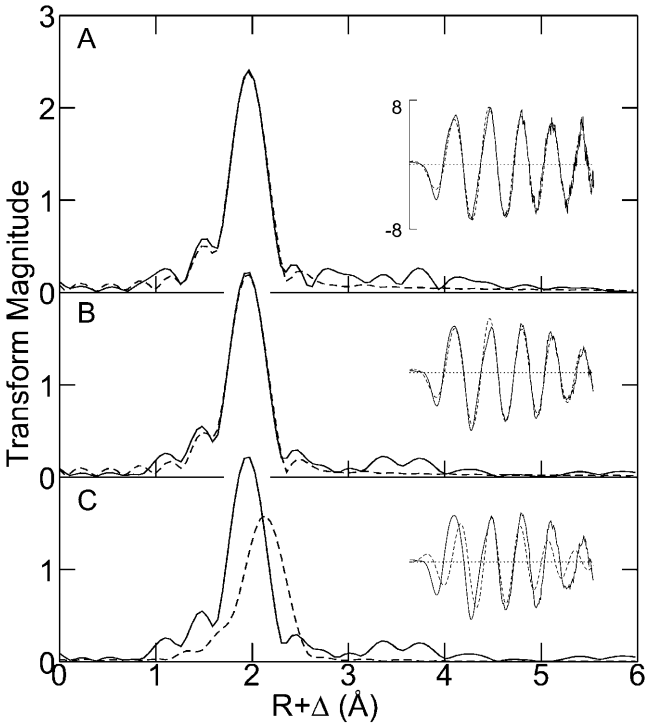

Figure 4. Ni K-Edge Extended X-Ray Absorption Fine Structure (Insets) and Fourier Transforms (Main) for Data and Fit.

Aqueous Ni nitrate **(A)** and field-collected *T. goesingense* leaf **(B)** fitted using the parameters shown in Table 1. Best fit of *T. goesingense* leaf to Ni-S shell **(C)**: 4 Ni-S at 2.24(1) Å, $\sigma^2 = 0.0096(18)$ Å^2; clearly the fit is very poor. All Fourier transforms have been phase corrected for Ni-O. Extended x-ray absorption fine structure (EXAFS) are plotted as $\chi(k)\cdot k^3$ with a *k*-range of 1 to 12.2 Å^{-1}. Solid line, data; dashed line, fit.

Cloning of *T. goesingense* SAT

SAT was cloned from *T. goesingense* by identification of cDNAs capable of conferring Ni resistance when expressed in *Escherichia coli*. Of the 200,000 primary *E. coli* transformants isolated, ~2000 where identified as being able to grow on LB plates containing 4 mM Ni^{2+}. Sequence analysis and functional complementation of the Cys auxotrophic phenotype of the *cysE*$^-$ mutant in *E. coli* revealed that the majority of these clones contained cDNAs encoding cytoplasmic (TgSAT-c), mitochondrial (TgSAT-m), and chloroplast (TgSAT-p) isoforms of SAT. When expressed in *E. coli*, full-length cDNAs of all three SAT isoforms conferred approximately equal resistance to Ni (Figure 5). Overexpression of *A. thaliana* SAT-p was also found to confer Ni resistance in *E. coli* (data not shown).

Overexpression of SAT in *A. thaliana*

The *T. goesingense* mitochondrial isoform of SAT (TgSAT-m) was overexpressed in *A. thaliana*. SAT protein level, enzyme activity, and OAS accumulation were all found to closely correlate in high (S 4-9), medium (S 3-1), very low (S 5-4) expressing, and control lines (Figures 6A and 6B). Analysis of the downstream metabolic products of SAT also revealed a strong positive correlation between SAT expression levels and shoot Cys and total glutathione content (Figures 6C and 6D). The highest SAT expressing line S 4-9 was observed to contain sevenfold higher Cys and threefold higher total glutathione compared with the control plants, with S 3-1 showing intermediate levels of Cys and total glutathione and the very low expresser S 5-4 showing no significant differences.

Effect of SAT Overexpression on Ni Resistance in *A. thaliana*

After growth for 21 d on medium containing 100 μM Ni, the highest SAT expressing line (S 4-9) showed no significant symptoms of Ni toxicity (Figures 7A and 7B). This contrasts with the Ni toxicity symptoms observed in control plants, including major loss of chlorophyll and significant reductions in both root and shoot growth (Figures 7A and 7B), consistent with that observed earlier (Figures 3A and 3B). Quantification of Ni resistance in the different TgSAT-m overexpressing lines, using a modified metal disc assay (Figures 7C to 7E) (Persans et al., 2001) widely accepted in the microbiological literature, revealed significant differences in resistance to Ni in both shoots (Figure 7D) and roots (Figure 7E) in the different transgenic lines. Though such an assay procedure produces only a relative measurement of resistance, quantified as distance from a Ni-soaked disc, it produces highly reproducible results and allows measurement of resistance over a continuous gradient of Ni concentrations. From this data the distance from the disc that causes a 50% inhibition of seedling growth can be estimated (I_{50}). Based on this I_{50} value, overproduction of SAT in the highest expressing line (S 4-9) produces a fivefold increase in shoot Ni resistance and a 1.5-fold increase in root resistance. Ni resistance measured at the seedling stage using the disc assay also reflects the root and shoot Ni resistance observed in more mature plants (Figures 7A and 7B), with Ni resistance being more pronounced in shoots compared with root length. Differences in Ni resistance observed in the SAT overproducing lines also strongly correlate with total glutathione concentrations in shoots of each line (Figure 8A).

Table 1. Ni-O First Coordination Shell Fits for Ni K-Edge EXAFS[a]

Sample	N	R	σ^2
T. goesingense leaf[b]	5.9(5)	2.036(3)	0.0046(6)
A. thaliana S 4-9[c]	6.0(9)	2.044(6)	0.0057(15)
A. thaliana empty vector[c]	5.4(7)	2.052(5)	0.0048(12)
Aqueous Ni nitrate[b]	6.0(5)	2.044(3)	0.0040(6)

[a] First shell Ni-O coordination number (N), distance (R) (Å), and Debye-Waller factor (σ^2) (Å^2). The errors in the last digit(s), shown in parentheses after each value, are three times the estimated standard deviations determined from the fit.

[b] Sample collected from a natural population in Austria, *k*-range of 1 to 12.2 Å^{-1}.

[c] *k*-range of 1 to 10.2 Å^{-1}. A second shell at about 2.9 Å was also included in these fits.

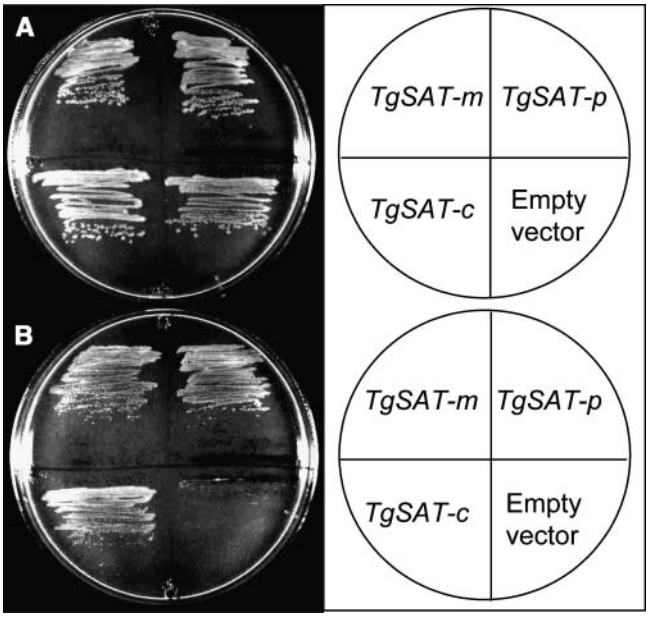

Figure 5. Ni Resistance of *E. coli* Expressing *TgSAT-m*, *TgSAT-p*, *TgSAT-c*, and Empty Vector Control Growing on LB Ampercillin (Amp) Plates and LB Amp + 4 mM Ni-Acetate.

(A) LB amp plates.
(B) LB amp + 4 mM Ni-acetate.

Importantly, addition of OAS, the product of SAT, to the growth media also conferred Ni resistance (data not shown), mimicking the effect of SAT overproduction. Addition of the isomer *N*-acetyl-L-Ser did not confer Ni resistance (data not shown). Ni accumulation was also measured in the SAT overexpressing plants (S 4-9) and compared with control plants after growth on 100 μM Ni for 21 d. No significant differences in shoot Ni accumulation were observed (Figure 8B).

An inhibitor of glutathione biosynthesis was used to further link elevated glutathione directly to the enhanced Ni resistance observed in SAT overproducing plants. Inhibition of glutathione biosynthesis using buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthatase (Meister, 1988), almost completely eliminated the enhanced Ni resistance of the SAT overexpressing plants (Figure 9). In planta Ni coordination in *A. thaliana* overexpressing TgSAT-m and control lines was also measured using XAS and determined to be predominantly Ni-O, with no detectable thiol coordination (Table 1).

Effect of SAT Overexpression on Resistance to Ni-Induced Oxidative Stress

The increased concentration of total glutathione in SAT overproducing plants is strongly correlated with reduced total lipid peroxidation in shoots after exposure to Ni (Figure 10A). The highest SAT expressing line S 4-9, which accumulates the highest concentration of OAS, Cys, and glutathione, showed only a 25% increase in total shoot lipid peroxidation after exposure to Ni, compared with 150% increase in control plants, with the intermediate SAT expressing line S 3-1 showing intermediate levels of lipid peroxidation. Total glutathione accumulation also correlate with increased resistance to Ni-induced accumulation of ROS in root tips, measured as the number of brightly fluorescent root tips (Figure 10B) and fluorescent intensity (Figures 10C to 10F). After exposure to Ni, the majority of root tips in the highest SAT producing line S 4-9 showed no reactive oxygen–induced fluorescence (Figure 10B). By contrast, >90% of root tips of control plants showed high levels of reactive oxygen–induced fluorescence (Figure 10E), with intermediate SAT producing lines showing intermediate levels of fluorescence (Figures 10C and 10D).

DISCUSSION

Elevated OAS, Cys, and Glutathione Correlate with Ni Hyperaccumulation

OAS, Cys, and total glutathione concentrations are found to be strong predictors of Ni hyperaccumulation ability across several Thlaspi hyperaccumulator and nonaccumulator species (Figure 1), suggesting that enhanced sulfur assimilation and glutathione biosynthesis play an important role in Ni hyperaccumulation. Such associations are independent of the presence of elevated Ni in the soil, suggesting that the role of enhanced sulfur assimilation in Ni hyperaccumulation is constitutive rather than Ni inducible. Constitutive mechanisms of metal hyperaccumulation have previously been identified, including constitutive overexpression of the Zn transporters ZNT1 in *T. caerulescens* (Pence et al., 2000; Assunção et al., 2001), ZIP6 and ZIP9 in *A. halleri* (Becher et al., 2004; Weber et al., 2004), the putative metal pumps TgMTP1, ZTP1, AhMTP1, and HMA3 in *T. goesingense*, *T. caerulescens*, and *A. halleri* (Assunção et al., 2001; Persans et al., 2001; Becher et al., 2004; Weber et al., 2004), and nicotianamine synthase in *T. caerulescens* and *A. halleri* (Vacchina et al., 2003; Becher et al., 2004; Weber et al., 2004). Considering metal hyperaccumulators usually complete their entire life cycle on soils enriched in Ni or Zn, it is not unexpected that the traits required for metal hyperaccumulation in these environments are constitutive (Reeves and Baker, 1984; Boyd and Martens, 1998; Bert et al., 2000). This can be contrasted with the responses observed in plants that are not preadapted to metal exposure. This is exemplified by the phytochelatin (PC)-mediated Cd resistance response observed in *A. thaliana* and other metal nontolerant plants. In response to Cd exposure, PC biosynthesis is induced and the accumulated PCs bind and detoxify the metal (Cobbett and Goldsbrough, 2002). Importantly, this response is only observed after exposure to the metal, and PCs are not produced constitutively (Cobbett and Goldsbrough, 2002). By contrast, naturally metal tolerant plants do not overproduce PC as part of their defense mechanism against Cd, Co, Cu, Ni, or Zn (De Knecht et al., 1992; Schat and Kalff, 1992; Harmens et al., 1993; Salt et al., 1999; Ebbs et al., 2002; Schat et al., 2002). Instead, they display a set of

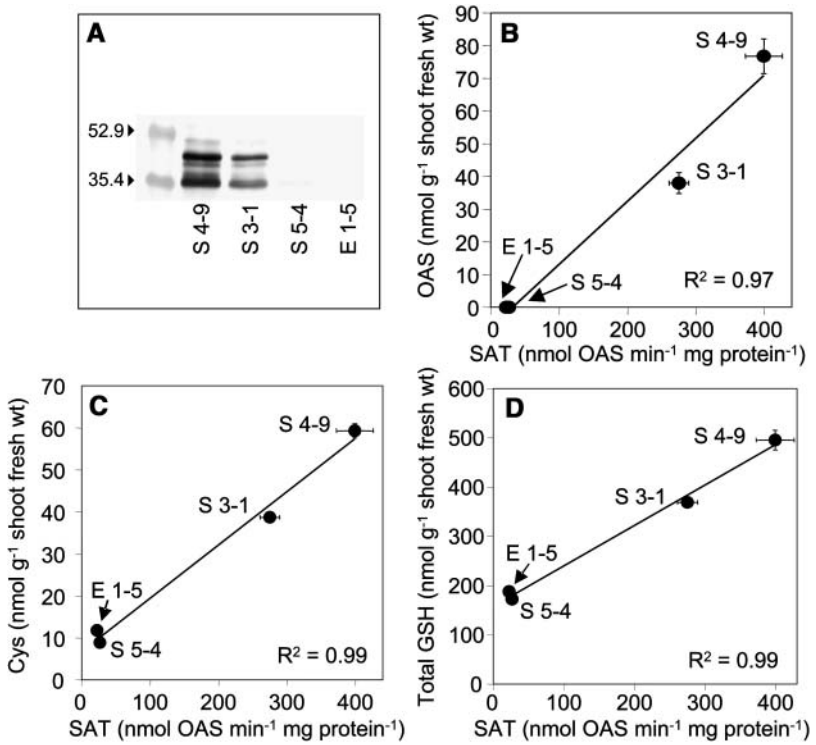

Figure 6. Effect on Sulfur Assimilation of SAT Overproduction in *A. thaliana* Shoot Tissue from Independent Homozygous T4 Lines Transformed with Vector Containing *TgSAT-m* (S 4-9, S 3-1, and S 5-4) or Empty Vector (E 1-5).

Immunoblot **(A)** decorated with polyclonal antibody raised against *A. thaliana* SAT-m. Correlation between shoot SAT activity and OAS **(B)**, Cys **(C)**, and total glutathione **(D)**. Data represent average of independent replicates ($n = 3$) ± SD. Trend line and R^2 represent regression analysis.

constitutive mechanisms including enhanced efflux and vacuolar compartmentalization (Chardonnens et al., 1999; Van Hoof et al., 2001).

To further establish the role of OAS, Cys, and glutathione in Ni hyperaccumulation, a more detailed comparative study of sulfur metabolism was undertaken. Ni tolerance and hyperaccumulation have been well studied in the Ni hyperaccumulator *T. goesingense* (Krämer et al., 1997, 2000; Küpper et al., 2001; Persans et al., 2001). Previously, many comparative physiological, biochemical, and molecular studies have been undertaken between the hyperaccumulators *T. goesingense* and *T. caerulescens* and the nonaccumulating relative *T. arvense*. In these studies, the choice of nonaccumulator was usually based more on availability rather than any strong phylogentic relationship. However, when studies were performed in other nonaccumulating species, including *A. thaliana* and *Brassica juncea*, similar findings were observed; for example, constitutive overexpression of *ZTP1* and *TgMTP1* in *T. caerulescens* and *T. goesingense* (Assunção et al., 2001; Persans et al., 2001). Because of the reorganization of the Brassicaceae family, based on ribulose-1,5-bisphosphate carboxylase/oxygenase, ITS nuclear ribosomal DNA, and chloroplast DNA restriction-site variation (Mummenhoff and Zunk, 1991; Mummenhoff and Koch, 1994; Zunk et al., 1996; Mummenhoff et al., 1997), *T. goesingense* along with many other Thlaspi metal hyperaccumulators have been moved into the Noccaea genus and the nonaccumulator *T. perfoliatum* moved into Microthlaspi, whereas *T. arvense* has remained in Thlaspi. It is now quite clear that the commonly used nonaccumulating relative *T. arvense* is not significantly more closely related to the hyperaccumulating Thlaspi (Noccaea) than the model plant *A. thaliana*. For example, the sequence of internal transcribed spacers of nuclear ribosomal DNA (ITS1 and ITS2) of both *T. arvense* and *A. thaliana* show 86% identify to *T. goesingense*. Low OAS, Cys, and glutathione concentrations also occur in all five broadly related nonaccumulating species studied including *A. thaliana*, *A. lyrata*, *T. arvense*, *T. perfoliatum* (*Microthlaspi perfoliatum*), and *T. montanum* var fendleri (*Noccaea fendleri* subsp *montanum*) (Figure 1). These observations suggest that robust conclusions about the role of sulfur metabolism in Ni hyperaccumulation can be drawn from comparisons of any of the closely related Brassicaceae nonaccumulator and hyperaccumulator species. Based on this evidence, and the available genetic resources, *A. thaliana* makes an obvious choice as a comparative nonaccumulator. This choice allowed comparisons to be made between hyperaccumulator, nonaccumulator, and nonaccumulator overexpressing genes from the hyperaccumulator. Comparison of the ability of the hyperaccumulator *T. goesingense* and the nonaccumulator *A. thaliana* to grow on medium containing elevated concentrations of Ni clearly demonstrates the significantly elevated tolerance of *T. goesingense* to Ni (Figures 3A and 3B), observed previously in comparison with *T. arvense* (Krämer et al., 1997).

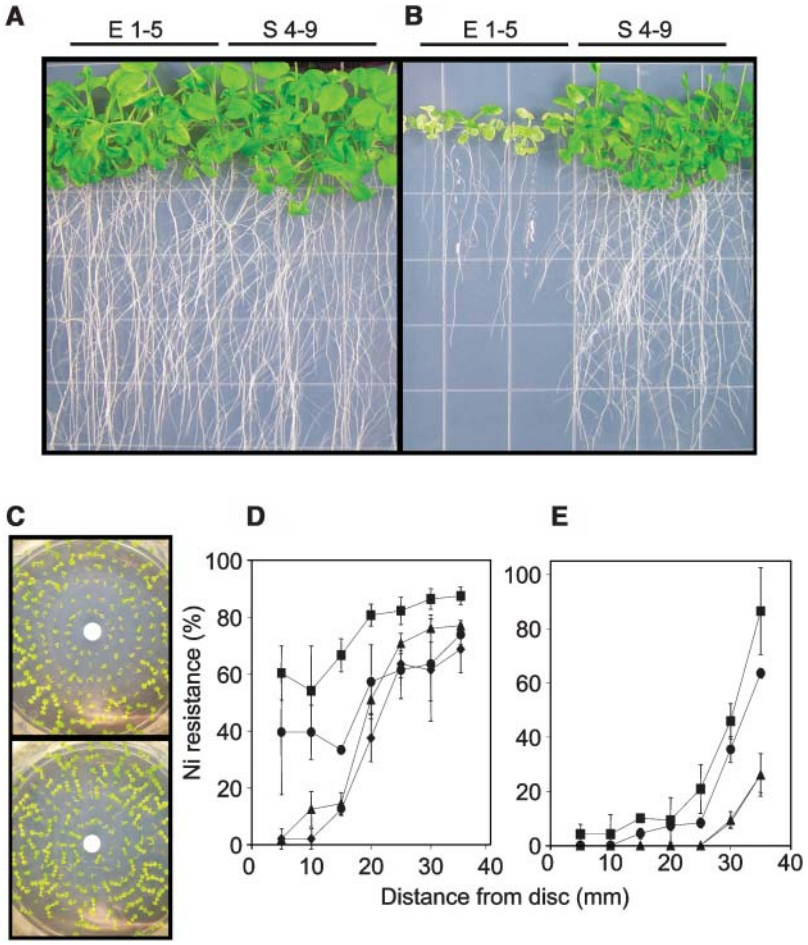

Figure 7. Effect on Ni Resistance of SAT Overproduction in *A. thaliana* Shoot Tissue from Independent Homozygous T4 Lines Transformed with Vector Containing *TgSAT-m* (S 4-9, S 3-1, and S 5-4) or Empty Vector (E 1-5).

Plants were grown together on half-strength MS agar plates **(A)** or plates containing 100 μM Ni^{2+} **(B)** for 21 d from imbibition. Both empty vector control (E 1-5; **[C]**, top panel) and SAT overproducing (S 4-9, bottom panel) plants were spotted in concentric rings radiating from a central paper filter disk soaked with 100 μL of 100 mM $Ni(NO_3)_2$ and photographed after 13 d of growth. Number of seedlings in each ring with upright cotyledons **(D)** and root hooks formed at the bottom of the plate **(E)** were scored and plotted as a percentage of the total number of seedling in a given ring and labeled as Ni resistance (%) against distance from the disc. Data represent average of independent replicate plates ($n = 3$) ± SD.

Levels of SAT activity and Cys and glutathione in the hyperaccumulator *T. goesingense* are in the range of those observed in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) overexpressing AtSAT-m (Błaszczyk et al., 1999, 2002; Harms et al., 2000; Wirtz and Hell, 2002). Importantly, SAT activity or OAS concentrations are not further induced by Ni, confirming what had been suggested earlier about the constitutive nature of the relationship between OAS, Cys, and glutathione and Ni hyperaccumulation. Interestingly, SAT activity in the nonaccumulator *A. thaliana* was found to decline after 10 d of exposure to 10 μM Ni. It is important to note here that exposure to 50 μM Cd for 24 h has been shown to increase steady state levels of SAT mRNA in *A. thaliana* (Howarth et al., 2003). However, sustained elevation of SAT enzyme activity required for metal resistance was not observed. Based on immunoblotting, using an *A. thaliana* polyclonal antibody raised against AtSAT-m, shoot tissue of *T. goesingense* was found to contain equal levels of the SAT protein compared with *A. thaliana* (data not shown). However, *T. goesingense* does contain a fivefold increase in SAT activity as measured in a desalted whole shoot extract (Figure 2A). Assuming that the polyclonal SAT antiserum reacts with *A. thaliana* and *T. goesingense* SAT equally, the fivefold increase in SAT-specific activity observed in vitro in the hyperaccumulator could be because of either altered phosphorylation status or modified stability of the regulatory complex between SAT and OAS (thiol) lyase, both known to alter SAT activity in plants (Bogdanova and Hell, 1997; Yoo and Harmon, 1997). Reduction in the Cys sensitivity of one or more of the SAT isoforms in *T. goesingense* could also contribute to the increased in vivo SAT activity suggested by the elevated OAS in the

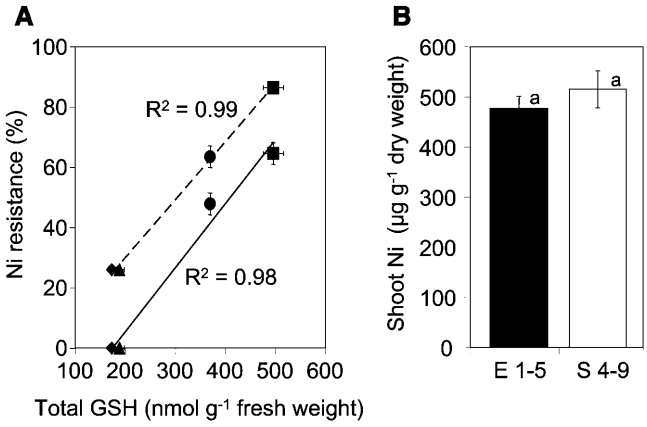

Figure 8. Relationship among Ni Resistance, Accumulation, and Glutathione in Shoot Tissue of Transgenic *A. thaliana* Overproducing SAT in Independent Homozygous T4 Lines Transformed with Vector Containing *TgSAT-m* (S 4-9, S 3-1, and S 5-4) or Empty Vector (E 1-5).

(A) Ni resistance was quantified as a percentage of seedlings with root hooks (dashed line) and upright cotyledons (solid line) scored in the ring showing the largest difference between the lines shown in Figure 7 and plotted against total shoot glutathione concentrations (E 1-5, diamonds; S 5-4, triangles; S 3-1, circles; S 4-9, squares). Data represent average of independent replicates ($n = 3$) ± SD. Trend line and R^2 represent regression analysis.

(B) Shoot Ni concentrations in empty vector control (E 1-5) and SAT overproducing (S 4-9) plants after growth for 21 d from imbibition on half-strength MS agar plates containing 100 μM Ni^{2+}. Data represent average of independent replicates ($n = 3$) ± SD. The same lower case letter (a) represents no significant differences between means (Student's *t* test, $P > 0.05$).

hyperaccumulators (Inoue et al., 1999). Comparative analysis of the predicted amino acid sequences of SAT from *T. goesingense* and *A. thaliana* reveals amino acid changes in both the predicted SAT/OAS (thiol) lyase interaction domain and the putative Cys binding domain (see Supplemental Figure 1 online; Bogdanova and Hell, 1997; Inoue et al., 1999). However, confirmation of the significance of such changes awaits future investigations into the enzymatic properties of SAT from *T. goesingense*, which are beyond the scope of this study.

Elevated SAT activity, giving rise to increased concentrations of OAS, Cys, and total glutathione, correlate with Ni hyperaccumulation in Thlaspi. However, the question remains as to what role such enhanced sulfur assimilation plays in Ni hyperaccumulation. Two major possibilities were investigated: either elevated glutathione gives rise to enhanced production of the Ni binding PC peptides (Cobbett, 2000), or elevated glutathione concentrations help protect against Ni-induced oxidative damage. Our direct measurement of the in planta binding environment of Ni in shoot tissue of *T. goesingense* collected from plants growing in their native habitat revealed that Ni is not bound by PCs in *T. goesingense* (Figure 4, Table 1), confirming in field collected plant tissue what was observed earlier on laboratory grown plants (Krämer et al., 2000). PCs have also been shown to play no role in Zn or Cd hyperaccumulation in *T. caerulescens* (Pickering et al., 1999; Ebbs et al., 2002).

Excess metals are known to confer oxidative damage in plants (Schützendübel and Polle, 2002), leading to lipid peroxidation and loss of membrane integrity. To protect against such damage, plants contain various enzymes designed to funnel superoxide radicals into H_2O_2 and H_2O through the action of superoxide dismutase, catalase, and ascorbate peroxidase. In this system, GSH acts as an intermediate reductant, funneling electrons from NADPH, via the action of glutathione reductase. As such, GSH plays a central role in this antioxidant mechanism. Elevated concentrations of GSH in the hyperaccumulator *T. goesingense* would be expected to help protect against Ni-induced oxidative damage. Such a protective mechanism is consistent with the observation that compared with the nonaccumulator *A. thaliana*, the hyperaccumulator *T. goesingense* suffers significantly less Ni-induced lipid perxidation in shoot tissue and accumulated ROS in root tips (Figures 3C to 3F). Further enhancing this oxidative stress resistance, the hyperaccumulator has elevated levels of both glutathione reductase and catalase, both enzymes involved in oxidative stress resistance. Interestingly, glutathione reductase activity and GSH levels have been found to be positively correlated in various plants (Mullineaux et al., 1994; Foyer et al., 1995; Pilon-Smits et al., 2000). These observations are also consistent with the previous observation that hairy root cultures of the Ni hyperaccumulator *A. bertolonii* have constitutively elevated catalase activity and a reduced sensitivity to Ni-induced lipid peroxidation compared with hairy roots of

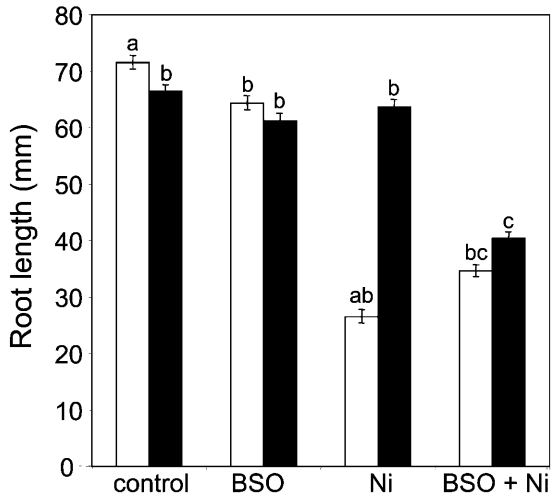

Figure 9. Effect of a Glutathione Biosynthetic Inhibitor on SAT-Mediated Ni Resistance.

Both empty vector control (E 1-5, open bar) and SAT overproducing (S 4-9, closed bar) *A. thaliana* seedlings were grown on half-strength MS agar plates containing either 100 μM BSO, 100 μM Ni^{2+}, or both BSO and Ni^{2+}. After 21 d from imbibition, length of the longest root on individual plants was measured. Data represent least squared means ± SE of root lengths of individual plants. Lower case letters (a, b, and c) represent significantly different means, using the mixed procedure function in SAS ($P < 0.01$). Experiment was set up as a randomized complete block design with three blocks ($n = 19 - 25$), and before analyses model assumptions where verified using residual analyses.

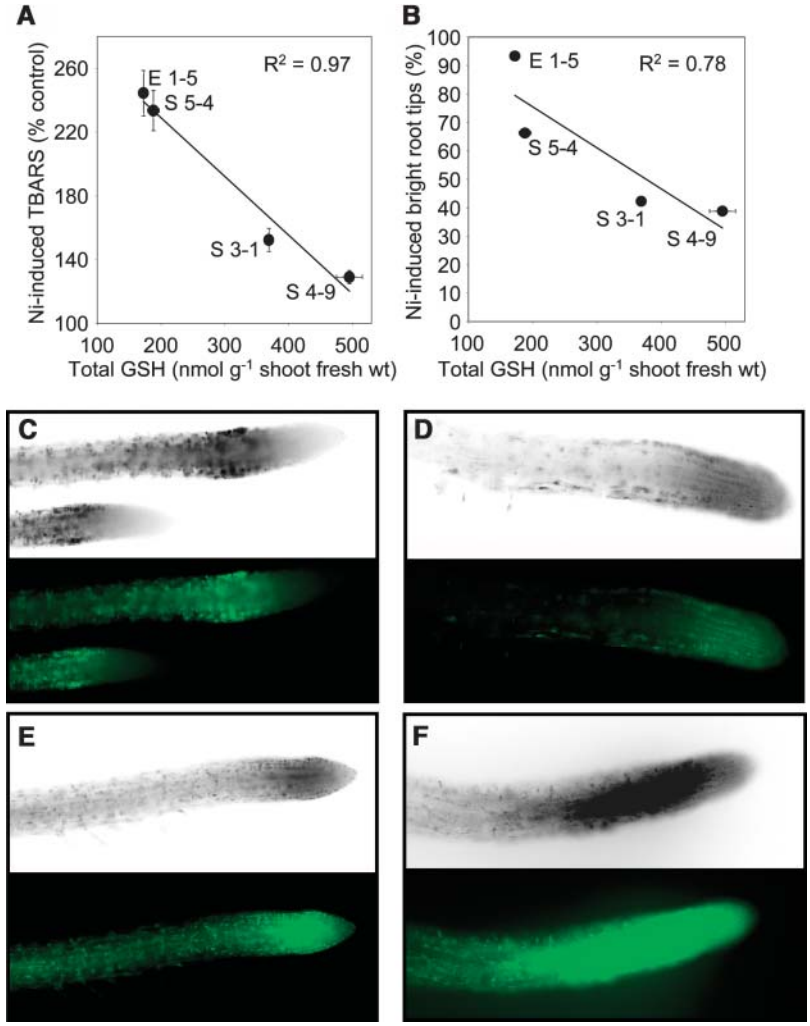

Figure 10. Effect of SAT Overproduction on Ni-Induced Oxidative Stress.

(A) and **(B)** Empty vector control and SAT overproducing *A. thaliana* were exposed to 125 μM Ni^{2+} for 7 d. Total shoot lipid peroxidation was measured as TBARS **(A)** and expressed as a percentage of TBARS measured in shoot tissue from lines grown in the absence of Ni. Root tip ROS were also measured as fluorescence of carboxy-H2DCFDA **(B)** and expressed as a percentage of total root tips scored (total tips scored: E 1-5 = 45, S 5-4 = 77, S 3-1 = 52, and S 4-9 = 67) and both TBARS and ROS plotted against total shoot glutathione. Data represent average ($n = 3$ total glutathione; $n = 4$ TBAR) ± SD. Trend line and R^2 represent regression analysis.

(C) to **(F)** Fluorescent images (positive and negative) of root tips of different *A. thaliana* lines overproducing SAT, including S 4-9 **(C)**, S 3-1 **(D)**, S 5-4 **(E)**, and empty vector control E 1-5 **(F)**.

N. tabacum (Boominathan and Doran, 2002). Interestingly, these authors reported that roots of the hyperaccumulator accumulated significant levels of H_2O_2 after Ni exposure, though the roots showed no symptoms of oxidative stress. From this the authors concluded that "*A. bertolonii* possesses detoxification mechanisms that allow unimpeded growth even in the presence of elevated concentrations of active oxygen species," though they did not speculate on what these mechanisms may be. Here, we present strong evidence that this oxidative stress defense mechanism involves constitutively elevated GSH concentrations, driven by enhanced SAT activity and maintained by enhanced glutathione reductase activity.

Elevated glutathione reductase and catalase activities are also found in a Ni-tolerant, Ni-accumulating strain of the green alga, *Scenedesmus acutus* f. *alternans* (Randhawa et al., 2001). Maize (*Zea mays*) tissue culture acclimated to high concentrations of Pb also show similar constitutively elevated glutathione reductase activity, which affords protection against Pb-induced oxidative damage (Zacchini et al., 2003). Glutathione reductase and catalase activities are also increased during Ni exposure in *Z. mays* shoots (Baccouch et al., 1998), a Ni-sensitive plant, suggesting that a similar, but less effective, Ni-inducible response may exist in other nonaccumulator plants. Altered superoxide dismutase (SOD) activity, however, appears not to play a significant role in

Ni tolerance in either alga (Randhawa et al., 2001) or *T. goesingense* (data not shown). Shoot tissue of the hyperaccumulator was also found to be tolerant to hydrogen peroxide, measured as loss of chlorophyll (data not shown), similar to that observed in *A. thaliana* overexpressing TgSAT-m (data not shown) and tobacco overexpressing bacterial SAT (Błaszcyk et al., 1999, 2002).

SAT Overexpression Enhances Ni Resistance in *A. thaliana*

Analyses of the predicted amino acid sequences of the processed form of the cloned TgSAT cDNAs after exclusion of the predicted targeting sequence (Noji et al., 1998) revealed high sequence identity between TgSAT-m, TgSAT-p, and TgSAT-c and their *A. thaliana* homologs At3g13110, At1g55920, and At5g56760. The mitochondrial form shows 95.0% identity, with the chloroplastic and cytoplasmic isoforms showing 85.5 and 98.1% identity, respectively (see Supplemental Figure 1 online). When overexpressed in *E. coli*, SAT conferred enhanced resistance to Ni, and this was used to clone SAT cDNAs from *T. goesingense*. Furthermore, overexpression of the cytoplasmic, mitochondrial, or chloroplastic SAT from *T. goesingense* or the chloroplastic SAT from *A. thaliana* in *E. coli* all conferred approximately equal Ni resistance. From this we can conclude that it is the enhanced SAT activity driving increased OAS biosynthesis that is important for Ni resistance in *E. coli*. In our experiments, this was achieved by driving gene expression from a strong promoter. Unique amino acid sequences in the SAT appear not to be required to achieve Ni resistance in *E. coli*.

To establish a direct link between glutathione and Ni hyperaccumulation, *T. goesingense* mitochondrial SAT was overexpressed in *A. thaliana*. The mitochondrial isoform of SAT (TgSAT-m) was chosen for overexpression in these studies because previously overexpression of the Cys-insensitive *A. thaliana* homolog AtSAT-m (SAT A) had been shown to achieve significantly elevated Cys and GSH concentrations when overexpressed in tobacco and targeted to either the plastid or the cytosol (Wirtz and Hell, 2002). Immunoblot analysis of SAT overexpressing lines revealed the presence of two major immunoreactive bands (Figure 6A). The higher molecular mass band likely represents the preprocessed form of the TgSAT-m protein with a predicted molecular mass of 43,447 D, whereas the lower band most likely represents the processed form with a predicted molecular mass of ~28,000 D, based on the predicted targeting sequence from *A. thaliana* (Noji et al., 1998). Such accumulation of the preprocessed form of SAT-m probably results from overloading of the mitochondrial import system because of high level expression of SAT (Wirtz and Hell, 2002). SAT enzyme activity in the TgSAT-m overexpressing lines of *A. thaliana* ranged from 275 to 400 nmoles OAS min^{-1} mg $protein^{-1}$ somewhat higher than that reported for transgenic tobacco overexpressing AtSAT-m, when activity was assayed directly as OAS production (Wirtz and Hell, 2002). Accumulation of OAS in shoots of TgSAT-m overexpressing *A. thaliana* ranged from 40 to 80 nmoles g^{-1} fresh weight, similar to that observed in *A. thaliana* grown under sulfate starvation conditions (Awazuhara et al., 2000). Because it is known that OAS concentrations regulate sulfur assimilation and Cys biosynthesis (Leustek, 2002; Hirai et al., 2003), we would expect that increased OAS concentrations would lead to increased accumulation of Cys and that is what we observed. In the TgSAT-m overexpressing *A. thaliana* lines, Cys concentrations in shoot tissue ranged from 40 to 60 nmoles g^{-1} fresh weight, similar to that observed previously in transgenic tobacco overexpressing *A. thaliana* AtSAT-m (Wirtz and Hell, 2002) or bacterial SAT in tobacco (Blaszczyk et al., 1999) or potato (Harms et al., 2000). Accumulation of the unprocessed, predicted Cys insensitive TgSAT-m isoform in the cytoplasm may help explain the high levels of SAT activity observed in these transgenic plants. Cys biosynthesis is one of the rate limiting steps in the biosynthesis of the antioxidant glutathione (Strohm et al., 1995; Noctor et al., 1996). By enhancing the rate of Cys biosynthesis in transgenic plants, we were able to increase glutathione concentrations in shoot tissue twofold to threefold, up to 500 nmoles g^{-1} fresh weight. Such elevated glutathione concentrations are comparable to those observed in the hyperaccumulators (Figures 1 and 2) and also obtained in tobacco overexpressing *A. thaliana* AtSAT-m (Wirtz and Hell, 2002) or bacterial SAT (Błaszczyk et al., 1999, 2002) and potato overexpressing bacterial SAT (Harms et al., 2000).

Overexpression of TgSAT-m in *A. thaliana* was found to confer significantly enhanced resistance to Ni measured as either shoot or root development, when assayed in plants germinated in the presence of Ni. Addition of OAS to the growth medium was also found to increase resistance to Ni, confirming that SAT overexpression was acting through enhanced levels of OAS. Addition of *N*-acetyl-L-Ser to the medium did not enhance Ni resistance, suggesting that metal resistance in this experiment was not because of Ni binding in the medium. This was further supported by the observation that shoot Ni concentrations in both Ni and Ni/OAS exposed plants were equal (data not shown). The lack of increased Ni accumulation supports the suggestion that glutathione concentrations play a role in Ni tolerance but not increasing Ni accumulation. Though both accumulation and tolerance are critical for hyperaccumulation, both are thought to be at least partly under independent genetic control (Macnair et al., 1999; Assunção et al., 2003; Bert et al., 2003). The positive correlation observed between shoot glutathione concentrations and Ni hyperaccumulation (Figure 1) does, however, suggest that at least among Ni hyperaccumulators collected from Ni-enriched environments, there is a link between internal Ni tolerance, mediated in part by glutathione, and shoot Ni accumulation, with the plants that accumulate the most Ni under native conditions having the highest level of glutathione and internal Ni tolerance.

Considering that the major biochemical effect of SAT overexpression is to elevate Cys and glutathione, we may expect that SAT overexpression would enhance the biosynthesis of PCs, thiol-rich peptides derived from GSH (Cobbett, 2000). The biosynthesis of PCs is known to be required for Cd resistance in *A. thaliana* (Howden et al., 1995), where they play a critical role in binding Cd via interactions with the Cys thiol groups (Strasdeit et al., 1991; Pickering et al., 1999). However, PCs are not involved in resistance to Ni, Co, and Zn in *A. thaliana* (Howden and Cobbett, 1992; Ha et al., 1999; Vatamaniuk et al., 2000; for review, see Cobbett, 2000). Furthermore, speciation of shoot Ni in TgSAT-m overexpressing *A. thaliana*, using XAS, directly confirmed Ni-O to be the predominant coordination form of Ni

with no significant presence of Ni-S (Table 1). Taken together, this evidence suggests that SAT overproduction is not influencing Ni resistance by enhanced PC synthesis.

Elevated glutathione in the transgenic plants overexpressing TgSAT-m acts to reduce Ni-induced oxidative damage, as suggested for the hyperaccumulators. Previously, increases in glutathione concentrations in tobacco, by overexpression of bacterial SAT or OAS (thiol) lyase from wheat (*Triticum aestivum*) or spinach (*Spinacia oleracea*) (Błaszczyk et al., 1999, 2002; Noji et al., 2001; Youssefian et al., 2001), have been shown to increase resistance to oxidative damage conferred by methyl viologen or hydrogen peroxide, and we also observed that *A. thaliana* overexpressing TgSAT-m showed elevated resistance to hydrogen peroxide (data not shown). However, this is the first report of increased glutathione biosynthesis conferring resistance to the damaging oxidative effects of Ni. This strong correlation between shoot glutathione concentration in the transgenic plants and their level of resistance to Ni provides strong support for our hypothesis that glutathione plays a role in Ni hyperaccumulation (Figure 1), acting to increase Ni tolerance in the hyperaccumulators.

Elevated SAT Activity Plays a Role in Ni Hyperaccumulation in Thlaspi

Evidence presented here, from both hyperaccumulator and transgenic *A. thaliana*, leads us to the conclusion that the elevated SAT activity and OAS, Cys, and glutathione concentrations observed in the hyperaccumulators play a causal role in Ni tolerance by enhancing the GSH-dependant antioxidant system. The constitutively elevated activities of GSH reductase and catalase in the hyperaccumulator also support such a conclusion. However, further work is required to determine the identity and localization of the SAT in Thlaspi responsible for elevated OAS concentrations that lead to increased Cys and glutathione. Investigations of the other enzymes involved in sulfur assimilation and glutathione biosynthesis are also necessary before we can fully understanding the biochemical basis of the enhanced glutathione levels observed in Thlaspi hyperaccumulators. It is also important to note that the glutathione mediated oxidative stress resistance mechanism in Thlaspi appears to represent only a part of the overall tolerance mechanism. It has been previously established that the vacuole provides a major sink for Ni in *T. goesingense* (Krämer et al., 2000; Küpper et al., 2001), and it has been suggested that this accumulation of Ni is driven by the constitutive overexpression of cation diffusion facilitator family members, including TgMTP1 (Persans et al., 2001). However, from 30 to 50% of cellular Ni in *T. goesingense* does appear to be localized outside the vacuole (Krämer et al., 2000). We propose that the glutathione-mediated antioxidant mechanism observed in *T. goesingense* and other hyperaccumulators plays a role in detoxification of this extravacuolar pool of Ni via enhanced oxidative stress resistance. Such a tolerance mechanism would be synergistic with nicotianamine-based chelation of Ni known to occur in hyperaccumulators (Vacchina et al., 2003). Stacking of genes that encode different components of the metal hyperaccumulation system in transgenic plants is now becoming a real possibility and should soon provide the ability to design plants ideally suited for the phytoextraction of pollutant metals.

METHODS

Plant Material

Thlaspi goesingense (Hálácsy) was collected from serpentine soil in Redschlag, Austria (Krämer et al., 1997), and *T. goesingense* shoot tissue from the same population was collected in the field, rapidly frozen on site, and shipped to the U.S. in dry ice for XAS analysis. Seeds for *T. montanum* var montanum from serpentine soil in Washington, U.S. (47°30′073″N, 121°03′407″W), *T. montanum* var fendleri from Schultz Pass, Flagstaff, AZ, and *T. arvense* from calcareous soil in Col de Gleize, France (44°37′164″N, 6°03′959″E) were also collected. All other accessions were as described in Peer et al. (2003) except seeds of *Arabidopsis thaliana* (Wassilewskija and Columbia-0), which were purchased from Lehle Seeds (Round Rock, TX). For analysis of OAS in a wide range of hyperaccumulator and nonaccumulator species, seeds were sown in soil and grown in the greenhouse as described in Peer et al. (2003). For all other experiments, *A. thaliana* (Wassilewskija) and *T. goesingense* were grown to the same developmental stage (5 and 7 weeks, respectively) in an artificial soil mix (Metro Mix TM; Scotts, Marysville, OH) in a growth room (24°C/20°C, 10 h/14 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux). For analyses, shoot tissue was harvested at the rosette stage 5 h after onset of the light period and immediately frozen in liquid nitrogen.

Metabolite Quantification

Frozen tissue was extracted in methanol at 4°C for 24 h, then phase extracted in water/chloroform at 4°C (Rhodes et al., 1986), with the addition of norvaline as an internal standard. Extracts were derivatized and analyzed using AccQ Tag following the manufacturer's instructions (Waters, Milford, MA). Extracts were resolved on an Alliance HPLC system (Waters) equipped with Millenium software, a 2695 separations module, a 2475 fluorescence detector, and a 2996 photodiode array detector. Standard curves were established using OAS (Sigma Chemical, St. Louis, MO; catalog number A-6262) and amino acid standard H (Pierce, Rockford, IL; catalog number NCI0180). Identity of OAS was confirmed by gas chromatography–mass spectrometry after methanol/chloroform/water extraction, standard amino acid fractionation (Rhodes et al., 1986), and derivatization with *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide in pyridine. Reduced and oxidized sulfur compounds were discriminated after *N*-ethylmaleimide protection (Lappartient and Touraine, 1997) and quantified on a Waters HPLC (Tsakraklides et al., 2002). Thiols were derivatized with monobromobimane and quantified as described (Tsakraklides et al., 2002).

Cloning of Genes Encoding SAT and Transformation of *A. thaliana*

The cDNAs encoding three different isoforms of SAT (GenBank accession numbers AY618468, AY618469, and AY618470) were cloned from a *T. goesingense* cDNA expression library (Persans et al., 1999) by the ability to confer tolerance to *Escherichia coli* growing on LB plates containing 4 mM Ni^{2+}. For overexpression studies, SAT cDNAs were cloned into pKYLX plant transformation vector behind the *Cauliflower mosaic virus* 35S promoter (An et al., 1985). *A. thaliana* was transformed using *Agrobacterium tumefaciens* by floral dipping (Clough and Bent, 1998) and transformed seeds selected on kanamycin. Empty pKYLX control lines were generated concurrently. Selected transformed lines were

allowed to self-fertilize and homozygous T3 and bulk T4 seed collected and used in all experiments.

Immunoblot Analysis

SDS-PAGE was performed as previously described (Laemmli, 1970). Crude extracts from all lines of transgenic *A. thaliana* overexpressing TgSAT-m were obtained by grinding tissue samples in liquid nitrogen and mixing the frozen powdered plant material in a 2:1 ratio (w/v) with SDS sample buffer. The mixture was boiled for 10 to 15 min and was centrifuged at 16,000*g*. The supernatant was assayed for total protein concentration using bicinchoninic acid (Pierce) and equal amounts of protein (30 μg) loaded onto an SDS-PAGE gel. For immunoblotting, proteins were transferred from the SDS-PAGE gel onto an immunoblot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using electrophoretic semidry blotting. SAT was visualized on the membrane using polyclonal primary antibodies raised against *A. thaliana* SAT-m in rabbits (kindly provided by Rüdiger Hell) and a secondary anti-immunoglobulin G antibody raised in goat and conjugated to alkaline phosphatase. Blots were developed by the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate. Immunoblots were also performed on *T. goesingense* and *A. thaliana* (Wassilewskija) (50 μg total protein loaded; $n = 12$ plants) (data not shown).

Enzyme Assays

Freshly frozen tissue was immediately assayed for SAT activity in Sepadex G-25 fine desalted plant extracts after homogenization in 100 mM KH_2PO_4/K_2HPO_4, pH 7.6, by quantification of *O*-acetylserine produced after addition of Ser and acetyl-CoA, with a final reaction mixture of 100 μL containing 1 mM L-Ser, 0.1 mM acetyl-CoA, 1.25 mM Na_2EDTA, 0.1 mM norvaline (internal standard) in 100 mM KH_2PO_4/K_2HPO_4, pH 7.6, at 25°C (Błaszczyk et al., 2002). Enzyme assays were stopped during the linear phase of the reaction at 20 min by the addition of ice cold methanol/chloroform (2:1 [v/v]) and stored overnight at −80°C. *O*-acetylserine in the aqueous phase was derivatized and quantified using AccQ Tag following the manufacturer's instructions (Waters). Glutathione reductase was assayed in desalted total plant extracts in 100 mM Na_2HPO_4 and 1 mM EDTA, pH 7.5, with HCl, using 5,5′-dithio-bis(2-nitrobenzoic acid) (Smith et al., 1988). Catalase activity was assayed in desalted plant extracts after homogenization in 50 mM KH_2PO_4/K_2HPO_4, pH 7.0, buffer containing 1% (w/v) polyvinylpolypyrrolidone, 1 mg/mL of DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/mL of pepstatin. Extracts were added to a 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0, and the decomposition of H_2O_2 measured at 240 nm (Lück, 1963). Superoxide dismutase was assayed in desalted plant extracts after homogenization in 50 mM KH_2PO_4/K_2HPO_4, pH 7.4, using a SOD assay kit following the manufacturer's instructions (SOD Assay Kit-WST; Dojindo Molecular Technologies, Gaithersburg, MD).

Quantitative Metal Tolerance Assays

Ni resistance in *A. thaliana* was tested on agar plates (100 × 25 mm) containing half-strength MS medium plus B5 vitamins. Seeds from SAT overproducing *A. thaliana* and empty vector control lines were spotted in concentric rings radiating from the center of the plate where a filter paper disk was placed. Onto the disk 100 μL of 100 mM $Ni(NO_3)_2$ was pipetted and the plates incubated at 24°C/20°C, 10 h/14 h light/dark, 120 μmol m^{-2} s^{-1}. After 13 d of growth from imbibition, the percentage of upright seedlings with fully expanded cotyledons, secondary leaves, and hooked roots were recorded for each ring along with the distance in millimeters from the Ni-soaked filter disk. Seedlings from rings 4 and 7 were then harvested for inductively coupled plasma mass spectroscopy analysis of metal levels. Metal analysis was performed on dried shoot tissue as previously described (Lahner et al., 2003).

Quantification of Total Lipid Peroxidation

Peroxidized lipids were assayed as the presence of TBARS. Plants were grown for 21 d under 10-h white light (120 μmol m^{-2} s^{-1} of photosynthetic photon flux) on vertical agar plates containing half-strength MS medium, then transfered to half-strength MS plates, containing 125 μM Ni, and shoot tissue was harvested and assayed for TBARS as described previously (Murphy et al., 1999). During the period of metal exposure, all plants remained viable and continued growing.

Imaging of ROS

The presence of ROS was determined in root tips of *T. goesingense*, *A. thaliana*, all transgenic *A. thaliana* lines overexpressing TgSAT-m, and empty vector controls. After 17 d of growth under 16-h white light (120 μmol m^{-2} s^{-1} of photosynthetic photon flux) on vertical agar plates containing half-strength MS medium, plants were transferred on media containing 125 μM Ni and allowed to grow for a further 8 d. Roots were stained with 10 μM carboxy-H2DCFDA (Molecular Probes, Eugene, OR) for 15 min and then rinsed. A Nikon Eclipse 3000 epifluorescent microscope (Melville, NY) equipped with a green fluorescent protein filter (excitation 450 to 490 nm, emission 500 to 530 nm) was used for epifluorescence images. Exposure times were equal for all samples. Autofluorescence was not observed in unstained controls at the exposure time used. Images were captured with a SPOT camera (Nikon).

XAS

Plant samples were analyzed following our previously described methods (Pickering et al., 1999). Briefly, samples for Ni K-edge XAS were shipped to Stanford Synchrotron Radiation Laboratory on dry ice, carefully ground under liquid nitrogen and compacted into Lucite sample holders with Mylar windows cooled in liquid nitrogen. During data collection, samples were held at ~15K using a liquid helium cryostat. Ni K-edge spectra were measured on beam line 7-3 using a Si(220) double crystal monochromator, 1-mm upstream vertical aperture, and no focusing optics, and the absorption spectrum collected in fluorescence using a 13-element germanium detector. XAS data reduction was performed using the EXAFSPAK suite of programs (http://ssrl.slac.stanford.edu/exafspak.html) and EXAFS phase and amplitudes calculated using the program *feff7* (Rehr et al., 1991).

Statistical Methods

All statistical analyses were performed using the SAS program (version 8e).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY618468, AY618469, and AY618470.

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REFERENCES

An, G., Watson, B.G., Stachel, S., Gordon, M.P., and Nester, E.W. (1985). New cloning vehicles for transformation of higher plants. EMBO J. **4,** 277–284.

Assunção, A.G.L., Martins, P.D.C., Folter, S.D., Vooijs, R., Schat, H., and Aarts, M.G.M. (2001). Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. **24,** 217–226.

Assunção, A.G.L., Ten Bookum, W.M., Nelissen, H.J.M., Vooijs, R., Schat, H., and Ernst, W.H.O. (2003). A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caesrulescens*. New Phytol. **159,** 383–390.

Awazuhara, M., Hirai, M.Y., Hayshi, H., Chino, M., Naito, S., and Fujiwara, T. (2000). *O*-acetyl-L-serine content in rosette leaves of *Arabidopsis thaliana* affected by S and N nutrition. In Sulfur Nutrition and Sulfur Assimilation in Higher Plants, C. Brunold, H. Rennenberg, L.J. De Kok, I. Stulen, and J.C. Davidian, eds (Bern, Switzerland: Paul Haupt), pp. 331–333.

Baccouch, S., Chaoui, A., and El Ferjani, E. (1998). Nickel-induced oxidative damage and antioxidant responses in *Zea mays* shoots. Plant Physiol. Biochem. **36**, 689–694.

Becher, M., Talke, I.N., Krall, L., and Krämer, U. (2004). Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J. **37,** 251–268.

Bert, V., Macnair, M.R., De Laguerie, P., Samumitou-Laprade, P., and Petit, D. (2000). Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). New Physiol. **146,** 225–233.

Bert, V., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N. (2003). Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. Plant Soil **249,** 9–18.

Błaszczyk, A., Brodzik, R., and Sirko, A. (1999). Increased resistance to oxidative stress in transgenic tobacco plants overexpressing bacterial serine acetyltransferase. Plant J. **20,** 237–243.

Błaszczyk, A., Sirko, L., Hawkesford, M.J., and Sirko, A. (2002). Biochemical analysis of transgenic tobacco lines producing bacterial serine acetyltransferase. Plant Sci. **162,** 589–597.

Bogdanova, N., and Hell, R. (1997). Cysteine synthesis in plants: Protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. **11,** 251–262.

Boominathan, R., and Doran, P.M. (2002). Ni-induced oxidative stress in roots of the Ni hyperaccumulator, *Alyssum bertolonii.* New Phytol. **156,** 205–215.

Boyd, R.S., and Martens, S.N. (1998). Nickel hyperaccumulation by *Thlaspi montanum* var. *montanum* (Brassicaceae): A constitutive trait. Am. J. Bot. **85,** 259–265.

Brooks, R.R., Lee, J., Reeves, R.D., and Jaffré, T. (1977). Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J. Geochem. Explor. **7,** 49–77.

Byers, H.G. (1935). Selenium occurrence in certain soils in the United States, with a discussion of related topics. U.S. Dept. Agric. Tech. Bull. **482,** 1–47.

Chardonnens, A.N., Koevoets, P.L.M., van Zanten, A., Schat, H., and Verkleij, J.A.C. (1999). Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. Plant Physiol. **120,** 779–785.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

Cobbett, C., and Goldsbrough, P. (2002). Phytochelatins and metallothioneins: Role in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. **53,** 159–182.

Cobbett, C.S. (2000). Phytochelatins and their role in heavy metal detoxification. Plant Physiol. **123,** 825–832.

De Knecht, J.A., Koevoets, P.L.M., Verkleij, J.A.C., and Ernst, W.H.O. (1992). Evidence against a role for phytochelatins in naturally selected increased cadmium tolerance in *Silene vulgaris* (Moench) Garcke. New Phytol. **122,** 681–688.

Ebbs, S., Lau, I., Ahner, B., and Kochian, L. (2002). Phytochelatins synthesis is not responsible for Cd tolerance in Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta **214,** 635–640.

Foyer, C.H., Souriau, N., Perrer, S., Lelandais, M., Kunert, K.-J., Pruvost, C., and Jouanin, L. (1995). Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in Poplar trees. Plant Physiol. **109,** 1047–1057.

Guerinot, M.L., and Salt, D.E. (2001). Fortified foods and phytoremediation: Two sides of the same coin. Plant Physiol. **125,** 164–167.

Ha, S.-B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B., and Cobbett, C.S. (1999). Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. Plant Cell **11,** 1153–1163.

Harmens, H., Hartog, P.R.D., Ten Bookum, W.M.T., and Verkleij, J.A.C. (1993). Increased zinc tolerance in *Silene vulgaris* (Moench) Garcke is not due to increased production of phytochelatins. Plant Physiol. **103,** 1305–1309.

Harms, K., von Ballmoos, P., Brunold, C., Hofgen, R., and Hesse, H. (2000). Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione. Plant J. **22,** 335–343.

Hell, R. (1997). Review: Molecular physiology of plant sulfur metabolism. Planta **202,** 138–148.

Hesse, H., Harms, K., Von Ballmoos, P., Brunold, C., Willmitzer, L., and Höfgen, R. (2000). Serine acetyltransferase: A bottleneck for cysteine and glutathione synthesis. In Sulfur Nutrition and Sulfur Assimilation in Higher Plants, C. Brunold, H. Rennenberg, L.J. De Kok, I. Stulen, and J.C. Davidian, eds (Bern, Switzerland: Paul Haupt), pp. 321–323.

Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M., and Saito, K. (2003). Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of *O*-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J. **33,** 651–663.

Howarth, J.R., Domínguez-Solís, J.R., Gutíerrez-Alcalá, G., Wray, J.L., Romero, L.C., and Gotor, C. (2003). The serine acetyltransferase family in *Arabidopsis thaliana* and the regulation of its expression by cadmium. Plant Mol. Biol. **51,** 589–598.

Howden, R., and Cobbett, C.S. (1992). Cadmium-sensitive mutants of *Arabidopsis thaliana*. Plant Physiol. **99,** 100–107.

Howden, R., Goldbrough, P.B., Anderson, C.R., and Cobbett, C.S. (1995). Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. **107,** 1059–1066.

Inoue, K., Noji, M., and Saito, K. (1999). Determination of the sites required for the alloseric inhibition of serine acetyltransferase by L-cysteine in plants. Eur. J. Biochem. **266,** 220–227.

Jaffré, T., Brooks, R.R., Lee, J., and Reeves, R.D. (1976). *Sebertia acuminate*: A hyperaccumulator of nickel from New Caledonia. Science **193,** 579–580.

Krämer, U., Pickering, I.J., Prince, R.C., Raskin, I., and Salt, D.E. (2000). Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol. **122,** 1343–1353.

Krämer, U., Smith, R.D., Wenzel, W., Raskin, I., and Salt, D.E. (1997). The role of nickel transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Hálácsy. Plant Physiol. **115,** 1641–1650.

Küpper, H., Lombi, E., Zhao, F.-J., Wieshammer, G., and McGrath, S.P. (2001). Cellular compartmentalization of nickel in the hyperaccumulator *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*. J. Exp. Bot. **52,** 2291–2300.

Lahner, B., Gong, J., Mahmoudian, M., Smith, E.L., Abid, K.B., Rogers, E.E., Guerinot, M.L., Harper, J.F., Ward, J.M., McIntyre, L., Schroeder, J.I., and Salt, D.E. (2003). Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana*. Nat. Biotechnol. **21,** 1215–1221.

Lappartient, A.G., and Touraine, B. (1997). Glutathione-mediated regulation of ATP sulfurylase activity, SO_4^{-2} uptake, and oxidative stress response in intact canola roots. Plant Physiol. **114,** 177–183.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277,** 680–685.

Leustek, T. (2002). Sulfate metabolism. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0017, http://www.aspb.org/publications/arabidopsis/.

Lück, H. (1963). A spectrophotometric method for the estimation of catalase. In Methods of Enzymatic Analysis, H. Bergmeyer, ed (New York: Academic Press), pp. 886–887.

Macnair, M.R., Bert, V., Huitson, S.B., Saumitou-Laprade, P., and Petit, D. (1999). Zinc tolerance and hyperaccumulation are genetically independent characters. Proc. R. Soc. Lond. B Biol. Sci. **266,** 2175–2179.

Meister, A. (1988). Glutathione metabolism and its selective modification. J. Biol. Chem. **263,** 17205–17208.

Minguzzi, C., and Vergnano, O. (1948). Il contenuto di nichel nelle ceneri di *Alyssum bertolonii* Desv. Mem. Soc. Tosc. Sci. Nat. Ser. A **55,** 49–77.

Mullineaux, P., Creissen, G., Broadbent, P., Reynolds, H., Kular, B., and Wellburn, A. (1994). Elucidation of the role of glutathione reductase using transgenic plants. Biochem. Soc. Trans. **22,** 931–936.

Mummenhoff, K., Franzke, A., and Koch, M. (1997). Molecular phyogenetics of *Thlaspi* s.l. (Brassicaceae) based on chloroplast DNA restriction site variation and sequences of the internal transcribed spacers of nuclear ribosomal DNA. Can. J. Bot. **75,** 469–482.

Mummenhoff, K., and Koch, M. (1994). Chloroplast DNA restriction site variation and phylogenetic relationships in the genus *Thlaspi* sensu lato (Brassicaceae). Syst. Biol. **19,** 73–88.

Mummenhoff, K., and Zunk, K. (1991). Should *Thlaspi* (*Brassicaceae*) be split? Preliminary evidence from isoelectric focusing analysis of Rubisco. Taxon **40,** 427–434.

Murphy, A.S., Eisinger, W.R., Shaff, J.E., Kochian, L.V., and Taiz, L. (1999). Early copper-induced leakage of K^+ from Arabidopsis seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol. **121,** 1375–1382.

Noctor, G., Strohm, M., Jouanin, L., Kunert, K.-J., Foyer, C.H., and Rennenberg, H. (1996). Synthesis of glutathione in leaves of transgenic poplar overexpressing γ-glutamylcysteien synthetase. Plant Physiol. **112,** 1071–1078.

Noji, M., Inoue, K., Kimura, N., Gouda, A., and Saito, K. (1998). Isoform-dependant differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J. Biol. Chem. **273,** 32739–32745.

Noji, M., Saito, M., Nakamura, M., Aono, M., Saji, H., and Saito, K. (2001). Cysteine synthase overexpression in tobacco confers tolerance to sulfur-containing environmental pollutants. Plant Physiol. **126,** 973–980.

Peer, W.A., Mahmoudian, M., Lahner, B., Reeves, R.D., Murphy, A.S., and Salt, D.E. (2003). Identifying model metal hyperaccumulating plants: Germplasm analysis of 20 Brassicaceae accessions from a wide geographical area. New Phytol. **159,** 421–430.

Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L., Lasat, M.M., Garvin, D.F., Eide, D., and Kochian, L.V. (2000). The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc. Natl. Acad. Sci. USA **97,** 4956–4960.

Persans, M.W., Nieman, K., and Salt, D.E. (2001). Identification of a novel family of metal transporters uniquely expressed in metal hyperaccumulating *Thlaspi goesingense* (Hálácsy). Proc. Natl. Acad. Sci. USA **98,** 9995–10000.

Persans, M.W., Xiange, Y., Patnoe, J.M.M.L., Krämer, U., and Salt, D.E. (1999). Molecular dissection of histidine's role in nickel hyperaccumulation in *Thlaspi goesingense* (Hálácsy). Plant Physiol. **121,** 1–10.

Pickering, I.J., Prince, R.C., George, G.N., Rauser, W.E., Wickramasinghe, W.A., Watson, A.A., Dameron, C.T., Dance, I.G., Fairlie, D.P., and Salt, D.E. (1999). X-ray absorption spectroscopy of cadmium phytochelatin and model systems. Biochim. Biophys. Acta **1429,** 351–364.

Pilon-Smits, E.A.H., Zhu, Y.L., Sears, T., and Terry, N. (2000). Overexpression of glutathione reductase in *Brassica juncea*: Effects on cadmium accumulation and tolerance. Physiol. Plant **110,** 455–460.

Randhawa, V., Zhou, F., Jin, X., Nalewajko, C., and Kushner, D. (2001). Role of oxidative stress and thiol antioxidant enzymes in nickel toxicity and resistance in strains of the green alga *Scenedesmus acutus* f. alternans. Can. J. Microbiol. **47,** 987–993.

Reeves, R.D., and Baker, A.J.M. (1984). Studies on metal uptake by plants from serpentine and non-serpentine populations of *Thlaspi goesingense* Hálácsy (Cruciferae). New Phytol. **98,** 191–204.

Reeves, R.D., and Baker, A.J.M. (2000). Phytoremediation of toxic metals. In Using Plants to Clean Up the Environment, I. Raskin and B.D. Ensley, eds (New York: John Wiley & Sons), pp. 193–229.

Reeves, R.D., and Brooks, R.R. (1983). European species of *Thlaspi* L. (Cruciferae) as indicators of nickel and zinc. J. Geochem. Explor. **18,** 275–283.

Reeves, R.D., Schwartz, C., Morel, J.L., and Edmondson, J. (2001). Distribution and metal-accumulating behavior of Thlaspi caerulescens and associated metallophytes in France. Int. J. Phytorem. **3,** 145–172.

Rehr, J.J., Mustre de Leon, J., Zabinsky, S.I., and Albers, R.C. (1991). Theoretical X-ray absorption fine structure standards. J. Am. Chem. Soc. **113,** 5135–5140.

Rhodes, D., Deal, L., Haworth, P., Jamieson, G.C., Reuter, C.C., and Ericson, M.C. (1986). Amino acid metabolism of Lemna minor L. I. Responses to methionine sulfoximine. Plant Physiol. **82,** 1057–1062.

Sachs, J. (1865). Handbuch der Experimental-Physiologie der Pflanzen. Handbuch der Physiologischen Botanik. (W. Hofmeister, Engelmann, Leipzig), pp. 153–154.

Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., and Pickering, I.J. (1999). Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Env. Sci. Tech. **33,** 713–717.

Schat, H., and Kalff, M.M.A. (1992). Are phytochelatins involved in differential metal tolerance or do they merely reflect metal-imposed strain? Plant Physiol. **99,** 1475–1480.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., and Bleeker, P.M. (2002). The role of phytochelatins and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. J. Exp. Bot. **53,** 2381–2392.

Schützendübel, A., and Polle, A. (2002). Plant responses to abiotic stresses: Heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. **53,** 1351–1365.

Smith, I.K., Vierheller, T.L., and Thorne, C.A. (1988). Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). Anal. Biochem. **175,** 408–413.

Strasdeit, H., Duhme, A.-K., Kneer, R., Zenk, M.H., Hermes, C., and Nolting, H.-F. (1991). Evidence for discrete $Cd(SCys)_4$ units in cadmium phytochelatin complexes from EXAFS spectroscopy. J. Chem. Soc. Chem. Commun. **16,** 1129–1130.

Strohm, M., Jouanin, L., Kunert, K.-J., Pruvost, C., Polle, A., Foyer, C.H., and Rennenberg, H. (1995). Regulation of glutathione synthesis in leaves of transgenic polar (*Populus tremula* x *P. alba*) overexpressing glutathione synthetase. Plant J. **7,** 141–145.

Tsakraklides, G., Martin, M., Chalam, R., Tarczynski, M., Schmidt, M., and Leustek, T. (2002). Sulfate reduction is increased in transgenic Arabidopsis thaliana expressing 5-adenylylsulfate reductase from Pseudomonas aeruginosa. Plant J. **32,** 879–889.

Vacchina, V., Mari, S., Czernic, P., Marquès, L., Pianelli, K., Schaumlöffel, D., Lebrun, M., and Łobiński, R. (2003). Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectroscopy and electrospray MS/MS assisted by cloning using yeast complementation. Anal. Chem. **75,** 2740–2745.

Van Hoof, N.A., Koevoets, P.L., Hakvoort, H.W., Ten Bookum, W.M., Schat, H., Verkleij, J.A., and Ernst, W.H.O. (2001). Enhanced ATP-dependent copper efflux across the root cell plasma membrane in copper-tolerant *Silene vulgaris*. Physiol. Plant **113,** 225–232.

Vatamaniuk, O.K., Mari, S., Lu, Y.-P., and Rea, P.A. (2000). Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. J. Biol. Chem. **275,** 31451–31459.

Weber, M., Harada, E., Vess, C., Roepenack-Lahaye, E.V., and Clemens, S. (2004). Comparative microarray analysis of Arabidopsis thaliana and Arabidopsis halleri roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J. **37,** 269–281.

Wenzel, W.W., and Jockwer, F. (1999). Accumulation of heavy metals in plants grown on mineralized soils of the Austrian Alps. Environ. Pollut. **104,** 145–155.

Wirtz, M., and Hell, R. (2002). Production of cysteine for bacterial and plant biotechnology. Application of cysteine feedback-insensitive isoforms of serine acetyltransferase. Amino Acids **24,** 195–203.

Yoo, B.C., and Harmon, A.C. (1997). Regulation of recombinant soybean serine acetyltransferase by CDPK. Plant Physiol. **114** (suppl.), 267.

Youssefian, S., Nakamura, M., Orudgev, E., and Kondo, N. (2001). Increased cysteine biosynthesis capacity in transgenic tobacco over expressing an *O*-acetylserine(thiol) lyase modifies plant responses to oxidative stress. Plant Physiol. **126,** 1001–1011.

Zacchini, M., Rea, E., Tullio, M., and de Agazio, M. (2003). Increased antioxidative capacity in maize calli during and after oxidative stress induced by a long lead treatment. Plant Physiol. Biochem. **41,** 49–54.

Zunk, K., Mummenhoff, K., Koch, M., and Hurka, H. (1996). Phytogenetic relationships of *Thlaspi* s.l. (subtribe Thlaspidinae, Lepidieae) and allied genera based on chloroplast DNA restriction-site variation. Theor. Appl. Genet. **92,** 375–381.